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Review

Cellular factors in plant virus movement: At the leading edge of macromolecular trafficking in plants

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ABSTRACT

To establish systemic infection, plant viruses must be localized to the correct subcellular sites to accomplish replication and then traffic from initially infected cells into neighboring cells and even distant organs. Viruses have evolved various strategies to interact with pre-existing cellular factors to achieve these functions. In this review we discuss plant virus intracellular, intercellular and long-distance movement, focusing on the host cellular factors involved. We emphasize that elucidating viral movement mechanisms will not only shed light on the molecular mechanisms of infection, but will also contribute valuable insights into the regulation of endogenous macromolecular trafficking.

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Introduction

Viruses are obligate parasites that can only multiply their genomes in their host organisms. A successful infection requires counteracting host defenses as well as intimate interactions between the viral genomes/genome-encoded products and host cellular factors including the host transcriptional, translational, and macromolecular trafficking machineries. This review addresses viral intracellular, intercellular and systemic movement in plants, focusing on recent advances concerning the identification of host cellular factors involved in these processes.

The importance of studying viral movement mechanisms extends beyond the obvious practical applications such as development of antiviral methods. It has contributed to our discovery of basic principles of biology. A brief account of some milestones drives this point home. Molecular characterization of the trafficking mechanisms began with the groundbreaking discoveries that the 30 kDa movement protein

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(MP) encoded by *Tobacco mosaic virus* (TMV) was required for viral cell-to-cell movement (Deom et al., 1987; Meshi et al., 1987) and that the TMV MP was able to modify the size exclusion limit (SEL) of plasmodesmata (PD) (Wolf et al., 1989). This prompted subsequent rapid identification of MPs and other viral proteins required for or involved in the cell-to-cell and long-distance movement of many viruses [reviewed in (Lucas, 2006; Scholthof, 2005; Taliansky et al., 2008)]. Further studies revealed that recombinant viral MPs and RNAs could traffic cell-to-cell when delivered by microinjection (Fujiwara et al., 1993; Noueiry et al., 1994; Waigmann et al., 1994). This led to the hypothesis that plants have evolved mechanisms for PD-mediated cell-to-cell trafficking of proteins and RNAs, which was supported by the direct demonstration of cell-to-cell trafficking of microinjected maize transcription factor KNOTTED1 and its mRNA (Lucas et al., 1995). A variety of experimental approaches have now established that many plant transcription factors and RNAs traffic from cell to cell or from organ to organ, with some shown to regulate developmental processes (Giakountis and Coupland, 2008; Hannapel, 2010; Kehr and Buhtz, 2008; Turgeon and Wolf, 2009). In a recent example, directional trafficking of a protein and an miRNA was elegantly shown to regulate root development in *Arabidopsis thaliana* (Carlsbecker et al., 2010). These findings establish macromolecular trafficking as a new paradigm of gene regulation at the whole plant level.

To understand the mechanisms of macromolecular trafficking requires identification of the molecular components of the trafficking machinery. Research on viral movement has once again contributed important insights. Extensive studies have provided strong evidence for the role of the cytoskeleton, membrane systems and other factors in the intra- and intercellular trafficking of viral proteins and RNAs. We summarize these findings and discuss knowledge gaps as well as emerging areas for future investigations. We emphasize the importance of integrating experimental approaches to tackle fundamental problems and merging virological and cellular perspectives to advance conceptual development.

Intracellular virus movement

Following entry into a host cell, a virus must replicate itself and move to the cell periphery in order to transfer its nucleic acids and/or proteins into adjacent cells. These viral components further move into the vascular tissue for long-distance movement to establish systemic infection (Fig. 1). For many viruses, replication occurs at cellular membranes (Netherton et al., 2007; Sanfaçon, 2005). Certain DNA viruses replicate within the nucleus (Gutierrez, 1999; Lazarowitz et al., 2004) while in other cases, viruses replicate in cytoplasmic inclusions that contain both viral and host factors. For example, both TMV (an RNA virus) and *Cauliflower mosaic virus* (CaMV) (a DNA virus) form large inclusions that are believed to support virus replication (Heinlein et al., 1998; Mas and Beachy, 1999; Modjtahedi et al., 1984). However, regardless of where replication takes place, a virus must move from the site of replication into surrounding cells to mount a successful infection. Here we will present recent advances in the study of the role of the cytoskeleton and associated motor proteins in virus movement. However, it should be noted that viruses may also utilize the host endomembrane system for intracellular movement. For a more complete discussion of the role of membranes in intracellular movement see the reviews by Boevink and Oparka (2005) and Harrie et al. (2010).

The majority of early studies on plant virus intracellular movement focused on the association between the TMV MP with microtubules (Heinlein et al., 1995; McLean et al., 1995; Padgett et al., 1996). In fact, the TMV MP binds very strongly to microtubules and even contains motifs characteristic of tubulin (Boyko et al., 2000). Recent work has suggested that microtubule dynamics at the leading edge of infection may drive movement of MP, possibly in association with viral RNA, to the PD (Boyko et al., 2007; Brandner et al., 2008; Ouko et al., 2010; Sambade et al., 2008). In support of this idea, tobacco mutants defective in microtubule dynamics are less susceptible to TMV infection (Ouko et al., 2010) and pharmacological inhibition of

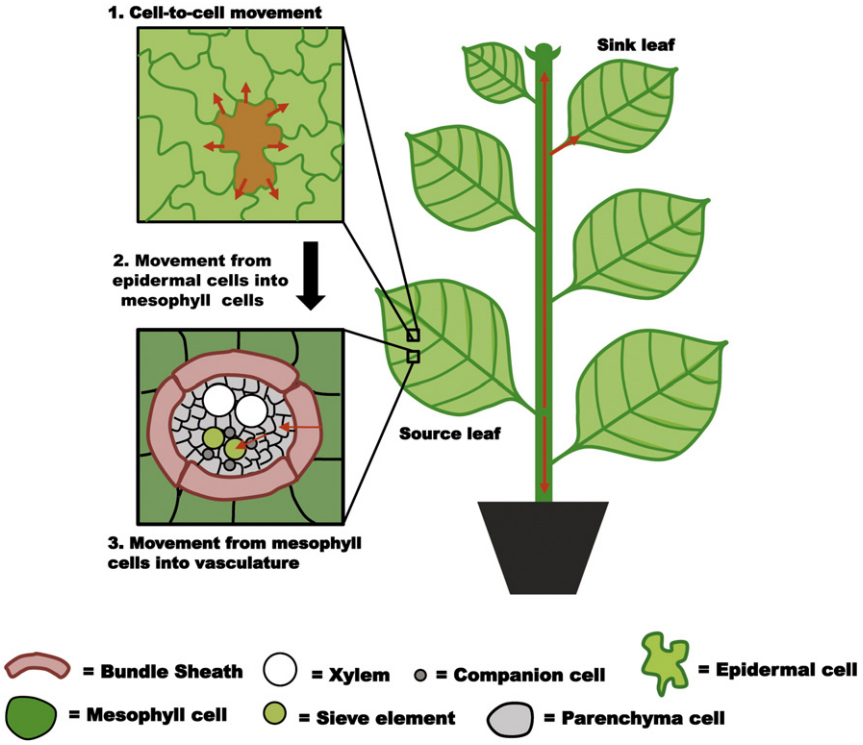

Fig. 1. Pathway for virus infection. Following entry of a virus into a host plant (source), in this case into a leaf epidermal cell, the virus must move cell-to-cell if the infection is to spread (1). In order for the virus to spread systemically, it must invade the mesophyll cells below the epidermis (2) and pass through bundle sheath, parenchyma and companion cells before finally entering the sieve element (3). Once the virus has gained access to the phloem, it may be transported to other leaves (sink) where the process of cell-to-cell movement may begin anew.

microtubule polymerization reduces the intracellular movement of TMV MP (Sambade et al., 2008). However, there are reports disputing the significance of MP-microtubule association for TMV movement. For instance, disruption of microtubule structure and/or polymerization by pharmacological treatments or by silencing the tubulin gene had no negative effect on TMV spread (Gillespie et al., 2002; Kawakami et al., 2004). Virus spread was also uninhibited by TMV MP mutants that had a reduced affinity for microtubules (Gillespie et al., 2002). Late in the infection process the TMV MP localizes to microtubules but does not move (Ashby et al., 2006; Boyko et al., 2007) suggesting that this could be part of a degradation pathway although the microtubule-associated MP is not ubiquinated (Ashby et al., 2006). Further work will clearly be required to resolve the differences between these studies. Although a number of other plant viral proteins also associate with microtubules, there is no clear evidence yet that such association is critical for virus movement [reviewed in (Benitez-Alfonso et al., 2010; Harrie et al., 2010)]. For example, recent work demonstrated that *Potato mop-top virus* (PMTV) triple-gene block protein, TGB1, interacts with microtubules. However, this association was only observed in cells behind the leading edge of the infection and lesion growth was not inhibited by treatment with microtubule disrupting inhibitors suggesting that the interaction between TGB1 and microtubules is not necessary for virus spread (Wright et al., 2010).

A number of recent studies have strongly implicated actin microfilaments in the intracellular movement of certain viruses. For example, large multi-component inclusions termed viral replication complexes (VRCs) formed in TMV infected cells localize to and traffic along microfilaments (Liu et al., 2005). VRCs were observed at the cell periphery, leading to the suggestion that they move along microfilaments to deposit viral components at the PD for intercellular transport (Kawakami et al., 2004). Although a role for VRCs in intercellular movement remains speculative, the idea is supported by the findings that intact microfilaments are required for TMV intercellular spread (Harries et al., 2009b; Kawakami et al., 2004). However, it should be pointed out that there is conflicting evidence suggesting that microfilaments are not required for TMV spread (Hofmann et al., 2009). In addition to TMV VRCs, both *Turnip mosaic virus* replication complexes and CaMV P6 inclusions can traffic along microfilaments (Cotton et al., 2009; Harries et al., 2009a).

The movement of these viral components along actin filaments suggests involvement of the myosins, a large superfamily of microfilament-associated molecular motors. All higher plant myosins are divided into two classes (VIII and XI) based on sequence differences in their conserved actin binding domains (Hodge and Cope, 2000; Lee and Liu, 2004). Recently two studies have begun to explore the significance of myosins for plant virus movement. *Beet yellows virus* requires five viral proteins for movement including the heat shock protein homolog, Hsp70h (Peremyslov et al., 1999). Hsp70h localizes to PD in a microfilament- dependent fashion, where it presumably facilitates the transfer of viral components from one cell to the next (Prokhnevsky et al., 2005). To determine a possible role of myosin in the intracellular movement of Hsp70h, Avisar et al. (2008a) ectopically expressed dominant negative myosins, which constained the cargo-binding tail domains but lacked the actin-binding head domains necessary for movement, in *Nicotiana benthamiana*. Expression of the tail domains of three different myosin. VIII's inhibited the localization of Hsp70h to PD whereas expression of the tail domains from three myosin XI's did not (Avisar et al., 2008a). Interestingly, the localization of TMV MP to the cell periphery was not inhibited in the presence of myosin VIII tails. These findings are particularly significant because they suggest different viruses may interact with distinct classes of myosins for movement.

Taking an alternative approach, Harries et al. (2009b) silenced specific *N. benthamiana* myosins via virus induced gene silencing (VIGS) and examined the consequence on the movement of diverse viruses. They found that silencing of myosin XI-2 specifically inhibited the movement of TMV but not that of *Potato virus X* (PVX) or *Tomato bushy stunt virus*, both of which were shown to require intact microfilaments for their spread (Harries et al., 2009b). Additionally, the closely related tobamovirus, *Turnip vein clearing virus* (TVCV), was also unaffected by myosin XI-2 silencing. Surprisingly, TVCV did not require microfilaments for movement. These findings expand upon those of Avisar et al. (2008a) to further demonstrate that individual viruses may utilize distinct subsets of myosins, even within a single class, for their movement. The utilization of the actin-myosin system by plant viruses for movement appears quite unique since animal viruses primarily utilize microtubules and their associated motor proteins for intracellular movement (Greber and Way, 2006; Radtke et al., 2006). These same constructs used to analyze the role of myosin in intracellular virus transport have also been successfully utilized to analyze the intracellular trafficking of organelles (Avisar et al., 2008b; Prokhnevsky et al., 2005).

Although much information has emerged on virus intracellular movement, primarily due to advances in live cell imaging, it is still quite difficult to paint a clear picture of virus trafficking within the cell. One problem is that although there are many studies demonstrating that viral proteins have particular subcellular localizations (ex. cytoskeleton or ER), the absence of follow-up studies to establish their clear roles in viral movement casts clouds on their biological significance. Given the potential importance of microfilaments in virus transport, the close association between the actin cytoskeleton and the ER makes it difficult to tease out the relative importance of each component. In fact, it was recently shown that myosin XI-K is responsible for the majority of ER streaming in plant cells and that if this myosin is inhibited, both ER organization and microfilament orientation are altered (Ueda et al., 2010). Also, the ER and the cytoskeleton are so important to normal cellular processes that testing the effects of disrupting these elements (pharmacological or otherwise) on virus movement may have unintended effects. In the future, it will be critical to utilize movement-deficient virus mutants as well as functional viruses with fluorescently tagged proteins (when possible) to help elucidate the virus-host interactions that are required for intracellular virus transport.

Intercellular virus movement

Although a distinction is generally made between intra- and intercellular movement, it may at times be difficult to separate the two. For example, since the ER network stretches from the cytoplasm, through the PD, and into the next cell, a virus may utilize this network for both intra- and intercellular transport. However, despite this continuity, the SEL of PD provides a physical barrier that must be overcome by a virus to successfully move from cell to cell. It is generally accepted that viral MPs increase the SEL [reviewed in (Harries and Nelson, 2008; Waigmann et al., 2004)]. Additionally, some MPs (in association with cellular components) form tubular structures through modified PD to allow viral movement (e.g., *Cowpea mosaic virus* and *Grapevine fanleaf virus*) (Laporte et al., 2003; Pouwels et al., 2003, 2004). A list of MPs and a more detailed account of their functions can be found in recent reviews by Scholthof (2005), Lucas (2006) and Harries and Nelson (2008).

Despite the importance of MPs in intercellular virus movement, there is relatively little information available about how they function to increase the PD SEL and how they, or other viral components, physically move through PD. The TMV MP was first shown to increase the PD SEL to allow intercellular movement of otherwise non-mobile cytoplasmic probes (Wolf et al., 1989), followed by similar discoveries with many other viral MPs [see review by (Lucas, 2006)]. Recent work suggests that the TMV MP can increase intercellular movement of fluorescent ER probes but not cytoplasmic probes (Guenoune-Gelbart et al., 2008). These findings led to different models of viral movement

through PD, which clearly require further experimental tests. Several studies suggest a possible mechanism by which MPs may increase the SEL. For example, experimental results with pharmacological treatments suggest that actin filaments play a role in controlling PD permeability (Ding et al., 1996) but it was not until recently that a strong link between MP activity and microfilaments was made. Su et al. (2010) utilized a biochemical approach to demonstrate that both the *Cucumber mosaic virus* (CMV) and TMV MPs inhibit actin polymerization and sever actin filaments. This microfilament-severing activity was required for MPs to increase the PD SEL. When microfilaments were stabilized by phalloidin or when MP mutants without severing activity were tested, no significant increase in PD SEL was observed (Su et al., 2010). Immunolabelling suggests localization of actin and myosin at PD (Overall and Blackman, 1996; Radford and White, 1998; White et al., 1994). Thus, it is conceivable that reorganization of the actin cytoskeleton by viral MPs results in a change of PD structure and a concomitant increase in the SEL. As a critical test of this model, whether actin and/or myosin are indeed localized to PD must be determined by alternative approaches such as biochemical isolation of these proteins from PD. It also must be demonstrated that all of these interactions have relevance for viral movement.

In addition to the actin cytoskeleton, the deposition of callose at the PD is also thought to be important for controlling permeability. Specifically, an increase in callose at the PD decreases the SEL (Radford et al., 1998; Radford and White, 2001; Sivaguru et al., 2000). When a β-1,3-glucanase (BG) capable of hydrolytic cleavage of callose was expressed from the TMV genome, viral spread was increased (Bucher et al., 2001). PVX requires three triple gene block (TGB) proteins and a coat protein (CP) for intercellular movement. One of these proteins, TGBp2, interacts in yeast two-hybrid screening with three host proteins termed TIPS (for TGB-interacting proteins) that in turn interact with BG (Fridborg et al., 2003). Although this finding suggests the possibility that TGBp2 recruits BG to the PD through an interaction with the TIP proteins to increase SEL, it remains to be demonstrated that such interactions occur *in vivo*, especially during viral infection. It must also be demonstrated that disruption of such interactions has a negative effect on viral movement. Using a similar yeast two-hybrid approach Lewis and Lazarowitz (2010) found that an *Arabidopsis* synaptotagmin (SYTA), a calcium sensor that regulates vesicle endo/exocytosis, interacts with the MPs of both TMV and *Cabbage leaf curl virus*. In SYTA mutant Arabidopsis plants, systemic infection is delayed and MP intercellular spread is inhibited (Lewis and Lazarowitz, 2010). The authors hypothesize that the viral MPs are directed to endosomal vesicles by their interaction with SYTA and that these vesicles then deposit the MP at the PD for intercellular movement. A similar endocytic pathway has also been proposed for PMTV proteins (Haupt et al., 2005). In contrast, it was recently shown that an exocytic pathway is used to deliver the *Turnip mosaic virus* (TuMV) movement-associated CI protein and P3N-PIPO proteins to the PD. The newly discovered P3N-PIPO protein was found to interact with CI and is believed to be important for the formation of conical structures at the PD that enhance virus movement (Wei et al., 2010).

Yeast two-hybrid screening also identified At-4/1, an *Arabidopsis* protein that interacts with the *Tomato spotted wilt virus* tubule-forming movement protein, NSm. At-4/1 localizes to PD and is capable of moving cell to cell (Paape et al., 2006), however, the biological relevance of these observations awaits additional experimental tests. PDLPs are another family of recently identified host proteins that localize to PD and interact with a tubule-forming MP. Recent work by Amari et al. (2010) demonstrated that PDLPs were important for the formation of tubules by the 2B MP from *Grapevine fanleaf virus* and that PDLP mutants slowed virus spread (Amari et al., 2010). Despite the growing wealth of information about virus-host interactions discussed above, additional studies are needed to identify other host proteins that interact with viral movement proteins. Recent successes with the two-hybrid approach such as the comprehensive work performed by Min et al. (2010) in which interactors of numerous *Sonchus yellow net virus* proteins were screened against a high resolution *N. benthamiana* yeast two-hybrid library and interactions validated by bimolecular fluorescence complementation illustrate the power of such an approach. Such findings will undoubtedly continue to increase our understanding of virus intercellular transport.

Viral proteins other than the classically defined MPs have increasingly been found to have movement capabilities. For example, both the TMV 126-kDa protein and the CaMV P6 proteins form motile bodies (Harries et al., 2009a; Liu et al., 2005). An outstanding question is whether and how these viral proteins interact with cellular factors as well as other viral components to promote viral movement. The potential interaction of such proteins with viral nucleic acids, either directly or indirectly, should be investigated since such interactions could have obvious implications for viral spread. Although the study of intercellular virus movement has made important contributions to our general understanding of PD structure and function (Lucas et al., 2009; Lucas and Lee, 2004; Oparka and Roberts, 2001; Overall, 1999), there remains much work to do in order to gain a clear understanding of the requirements for cell-to-cell movement of the huge diversity of plant viruses.

Long-distance movement

Viruses move long distance through the vascular tissue (mostly through phloem and in a few cases perhaps through the xylem). However, in contrast to the vast number of studies on intracellular and cell-to-cell movement, relatively few studies have specifically focused on host factors for phloem transport. The physical location of phloem deep within all organs of a plant body makes it challenging for experimental studies. Furthermore, attempts to separate the roles of host factors for long distance and cell-to-cell movement may not always be straightforward because of the inter-dependence of these two processes. Nonetheless, recent studies have begun to shed light on some factors critical for long-distance movement.

Earlier studies showed that a pectin methylesterase (PME) purified from tobacco leaves interacts with the MP of TMV and other viruses and that the interaction is required for TMV cell-to-cell movement (Chen et al., 2000). Antisense suppression of PME expression in tobacco led to delayed systemic movement of TMV (Chen and Citovsky, 2003). The open reading frame (ORF) 3 protein of *Groundnut rosette virus* (GRV), which has the unusual property of not encoding coat protein (CP), plays an essential role in viral RNA long distance movement (Ryabov et al., 1999). Upon entering the nucleus, the ORF3 protein interacts with and reorganizes Cajal bodies (CBs) that then fuse with the nucleolus. The nucleolar localization of the ORF3 protein is essential for long-distance movement (Kim et al., 2007b). Furthermore, the ORF3 protein causes redistribution of fibrillarin from the nucleus to the cytoplasm, which is required for long-distance movement since silencing fibrillarin inhibited viral movement (Kim et al., 2007a). These findings led to a model in which the ORF3 protein interacts with fibrillarin, enters the nucleolus through fibrillarin-mediated CB fusion, and then exits the nucleus to form 'transport-competent' viral RNP particles in the cytoplasm for long-distance movement (Kim et al., 2007a,b). Atomic force microscopy (AFM) revealed a ringlike structure of ORF3-fibrillarin complex formed *in vitro* (Canetta et al., 2008). This *in vitro* complex is infectious (Kim et al., 2007a) and a similar ringlike structure was observed in GRV-infected plants (Taliansky et al., 2003), supporting the biological relevance of the AFM structure. How this ringlike structure functions in viral movement remains to be further elucidated, but it is postulated that binding with ORF3 alters the structure of fibrillarin to facilitate viral movement (Canetta et al., 2008). In a recent study, interaction of the triple-gene-block (TGB)1 protein of *Potato mop-top virus* (PMTV) fused to yellow fluorescent protein with the nucleoli, and possibly also with microtubules, was shown to be critical for

systemic movement in *N. benthamiana*. Specifically, deletion of the 84 amino acids from the N terminus of unabled TGB1 abolishes systemic but not local cell-to-cell movement. The same deletion mutant fused to GFP also failed to interact with the nucleoli and microtubules. While the 84-aa N-terminal part is sufficient to interact with the nucleoli, it is not sufficient to interact with microtubules (Wright et al., 2010).

The CMV 1a protein, as a component of the viral replicase, is not only essential for viral replication but is also important for systemic movement (Palukaitis and Garcia-Arenal, 2003). Using yeast two-hybrid screening, Kim et al. (2008) identified a methyltransferase from tobacco, Tcoi1 (tobacco CMV 1a-interacting protein 1), that interacts with the methyltransferase domain of the CMV 1a protein. Furthermore, Tcoil methylates the 1a protein both *in vitro* and *in vivo*. Overexpression of Tcoi1 enhances CMV infection and inhibited expression of Tcoi1 decreases infection, without a strong impact on viral replication or cell-to-cell movement. Thus, the decreased viral infection associated with Tcoi1 silencing appears to be attributed to an inhibition of long-distance movement, although the specific mechanisms await further experimental investigations.

The proteosomes are also implicated in viral systemic movement. In a screen using VIGS, Jin et al. (2006) identified a 26S proteasome subunit, RPN9, required for viral systemic transport. Silencing RPN9 in *N. benthamiana* inhibited systemic spread of TMV and TuMV, likely as a result of altered development of the vasculature. RPN9 may function by regulating auxin transport and brassinosteroid signaling critical for vascular development. A more recent study suggests a role for protein degradation in viral phloem exit. Mekuria et al. (2008) generated transgenic *N. benthamiana* plants that express GFP fused to the PVX TGB p1 or the CP in companion cells. The fusion proteins were largely confined to the vasculature in petioles and leaf blades, indicating inability to exit the phloem. However, when GFP-tagged virus-infected leaves were treated with proteasome inhibitors, GFP fluorescence spread into mesophyll cells. These data suggest the intriguing possibility that proteolysis plays a role in restricting viral proteins in the phloem (Mekuria et al., 2008). Further molecular and genetic studies are required to test this model.

Genetic analysis of mutant host plants with compromised viral movement is an obvious approach but has been under-utilized. By infecting different ecotypes of *A. thaliana* exhibiting varying degrees of susceptibility to TMV strain U1 (TMV-U1), Serrano et al. (2008) showed that delayed TMV-U1 movement in ecotype Col-0 was correlated with a monogenic and recessive nuclear locus denoted DSTM1 (Delayed Systemic Tobamovirus Movement 1). Electron microscopy revealed curved virions in the stems of infected Col-0 plants in contrast to the typical rigid rod-like virions of TMV-U1 present in infected Uk-4 plants. Additionally, Gopalan (2007) identified an Arabidopsis mutant, B149, that did not support infection by *Tobacco etch virus* (TEV). This mutant was defective in initiation of infection foci, cell-to-cell and long distance movement. Cloning of DSTM1 and B149 should enable further mechanistic studies that will help clarify the role of these host proteins in systemic movement.

While identification of the host factors facilitating viral systemic movement is obviously critical to understand viral infection, it is equally important to understand negative regulators of movement. In general, host defense systems play a role in restricting systemic movement. Examples include defense mechanisms meditated by host RNA silencing (Cao et al., 2010), salicylic acid (Alamillo et al., 2006) and ethylene (Love et al., 2007). In particular, an RNA silencing-based surveillance mechanism may prevent invasion of shoot apical meristems by some viruses (Foster et al., 2002; Qu et al., 2005; Schwach et al., 2005).

Arabidopsis proteins RTM1 (Restricted TEV Movement1) and RTM2 function within the phloem to restrict viral long-distance movement (Chisholm et al., 2000, 2001; Mahajan et al., 1998; Whitham et al., 2000). Some virus isolates can break RTM resistance and recent studies showed that the N-terminal region of potyviruses is responsible (Decroocq et al., 2009). A future challenge is to elucidate the interactions between positive and negative factors that f control how a virus moves systemically within a plant.

Conclusions: moving into the future

A unique aspect of viral movement is the integration of processes from intracellular to whole plant levels. Thus, studies of virus movement will enable investigation of how basic cellular processes in individual cells may be coordinated in a whole plant through macromolecular trafficking. The role of nucleolar proteins in GRV movement provides a compelling example.

The reported roles of the cytoskeleton and membranes in trafficking are providing new insights to re-define our knowledge of basic plant cell biology as well as raising new questions.

The contrasting reports on the role of microtubules and microfilaments in viral movement highlight the need to continue developing innovative experimental approaches to resolve the differences. Many proposed models illustrate actin and myosin in PD. However, it should be borne in mind that the presence of these proteins in PD is mainly inferred from immunolabeling. It is imperative that additional approaches are used to test the model. It follows that major advances in our mechanistic understanding of cell-to-cell transport will require isolation of PD components and functional determination of their roles in viral movement.

While studies on the role of viral components for movement have predominantly focused on viral proteins, there is convincing evidence that viral RNAs also play a direct role, at least for viruses that have been studied (Chen et al., 2003; Choi et al., 2005; Gopinath and Kao, 2007; Lauber et al., 1998; Lough et al., 2006; Shin et al., 2008). Although the direct role of viral RNAs in movement remains to be further investigated, it may be postulated that distinct 3D RNA structural motifs are involved in interactions with host and/or viral factors for movement. The role of a 3D RNA motif in mediating intercellular movement has been demonstrated for *Potato spindle tuber viroid* (PSTVd), which does not encode any proteins (Zhong et al., 2007). Mutagenesis identified many PSTVd motifs, whose 3D structures remain to be determined, critical for replication as well as movement (Zhong et al., 2008). Thus, we suggest that investigating the direct role of viral RNAs in movement, including elucidating the viral and host factors interacting with potential RNA structural motifs, should be one of the major focuses for future investigations.

Given the overwhelming application of GFP fusions and confocal microscopy, it should not be forgotten that electron microscopy still has unparalleled resolution for protein localization studies. On the other hand, new experimental approaches must be adapted or developed. A significant lack of biochemical and genetic approaches has been a hurdle in mechanistic studies. Structural knowledge of viral proteins and host factors will also be necessary to obtain true knowledge of movement mechanisms. We should be mindful that, in order to make significant progress, cross-disciplinary collaborations will undoubtedly be required. Although it is obvious, it should be stressed that direct infection analysis must remain the basis for understanding the relevance of viral protein movement results in the context of systemic infection.

To gain a holistic picture of viral movement, it is imperative to probe deeper into the pathways. For instance, how does a virus move across distinct cellular boundaries? What are the common and unique regulations at different boundaries? How does defense play a role in this process? How are virus entry into and exit from the phloem controlled? What host factors are involved in movement across specific cellular boundaries and in the phloem? Are there factors that function commonly in intracellular, intercellular and long-distance movement? Elucidating these questions will not only provide fundamental insights into viral movement mechanisms, but will

surely shed light on the cellular machinery that regulates the trafficking of endogenous proteins and RNAs. The future of viral movement studies will be brighter when findings from these studies can be used to advance conceptual and technical advances for basic plant biology, beyond the boundaries of virology.

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